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DNA based one-pot TLAMP and PfAgo rapid assay biosensing platform for meat visualized authentication

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Abstract: Meat adulteration is a significant global food safety challenge, creating a pressing need for rapid and on-site detection technologies. Herein, we present an intelligent one-pot biosensing platform termed OTPA (One-pot TLAMP-PfAgo Assay) that integrates the rapid, isothermal amplification of turn-back loop primer-accelerated LAMP (TLAMP) with the sequence-specific detection of *Pyrococcus furiosus* Argonaute (PfAgo). This system features a clever heat-activatable design using microcrystalline wax to spatially separate reactions within a single tube, enabling contamination-free and streamlined operation. The OTPA assay achieves sensitive and specific detection, with limits of detection as low as 3×10^{-4} ng/μL for pork DNA and 2×10^{-4} ng/μL for beef DNA within 30 minutes. It successfully enables duplex target identification and has been validated with commercial meat products, showing perfect concordance with standard PCR-based qualitative detection. Notably, the result can be directly visualized under blue light, underscoring the substantial potential of this cost-effective and simple platform for point-of-care testing (POCT) and intelligent biosensing in food safety surveillance.

Key words: TLAMP; PfAgo; Meat authentication; One-pot assay; Point-of-care testing

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1 Introduction

Food safety is a critical issue globally, with meat adulteration posing significant threats to quality and safety (Li et al., 2023a). A primary concern is the substitution of high-value meats with cheaper alternatives, deceiving consumers and undermining industry integrity (Sofos, 2008; Montownska and Pospiech, 2010; Vlachos et al., 2016). Such fraud leads to economic losses and health risks, as evidenced by scandals like the 2013 horsemeat incident in Europe and the 2017 Brazil rotten meat case (Agnoli et al., 2016; Robson et al., 2020). Despite regulatory frameworks, fraudulent practices persist, impacting consumer health and security (Lianou et al., 2021).

Accurate discrimination of meat species is crucial for authentication. Conventional methods include morphological testing (Kamruzzaman et al., 2015), chromatographic analysis (Stachniuk et al., 2021), spectroscopic examination (Chaudhary et al., 2022), immunological analysis (Montowska and Pospiech, 2010),

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and DNA-based molecular testing (Adenuga et al., 2023). However, morphological methods are subjective, chromatographic techniques are labor-intensive and time-consuming, spectroscopic approaches face operational challenges for on-site use, and immunological assays lack sensitivity for trace detection. Recently, miniaturized Raman spectroscopy has gained great attention in this field due to its high portability (Müller-Maatsch and Van Ruth, 2021; Qu et al., 2022; He et al., 2024). Nevertheless, its performance is susceptible to spectral interference from the food matrix. Immunological analysis represents a rapid and cost-effective method for meat authentication, but insufficient sensitivity often leads to false-negative results when detecting trace amounts of target. In contrast, DNA-based molecular testing has become a pivotal tool in the identification of meat adulteration, as it not only offers high specificity but also sufficient sensitivity, enabling the detection of even minute quantities of adulterant species in meat products. Among DNA-based tools, polymerase chain reaction (PCR) is the gold standard (Fajardo et al., 2010) but requires thermal cycling and laboratory settings. Isothermal nucleic acid amplification (INAA) alternatives like loop-mediated isothermal amplification (LAMP) simplify workflows (Sheu et al., 2018; Yang et al., 2024) and are optimized for food safety (Sherry et al., 2015; Shufang et al., 2017; Shi et al., 2024). Recently, turn-back loop primer-accelerated LAMP (TLAMP) was developed for rapid amplification within 20 min (Shi et al., 2024), but depends on non-specific reporters, risking false positives.

Programmable nucleases have emerged as pivotal tools for nucleic acid manipulation, revolutionizing methodologies in nucleic acid testing. Beyond their well-established role in gene editing, the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-associated protein (Cas) system, collectively known as CRISPR/Cas, has been widely adopted in molecular diagnostics due to its sequence-specific trans-cleavage activity (Dai et al., 2020; Tian and Zhou, 2023). Nevertheless, the requirement for RNA-based guide molecules and the challenges associated with one-pot multiplex detection have limited the broader application of CRISPR/Cas systems. In recent years, prokaryotic Argonaute (pAgo) proteins, another class of programmable nucleases, have attracted growing attention owing to their distinct advantages over CRISPR/Cas systems (Yang et al., 2023; Li et al., 2024). Notably, pAgo uses DNA as a guide sequence, which is more cost-effective than RNA. Moreover, unlike the nonspecific trans-cleavage exhibited by activated CRISPR/Cas, pAgo enables precise cleavage of signal probes upon recognition by the guide DNA, thereby facilitating one-pot multiplex detection. These features have enabled successful applications of pAgo in pathogen and tumor DNA detection (Li et al., 2023b; Su et al., 2025). However, most reported approaches require manual lid-opening or centrifugation steps due to incompatibility between pAgo-mediated cleavage and amplification reactions (Liu et al., 2021). To address this limitation, in this study we integrated *Pyrococcus furiosus* Ago (PfAgo) with TLAMP to develop a novel meat identification method. To simplify detection and resolve reaction incompatibility, we introduced a heat-activated strategy using microcrystalline wax, enabling a fully integrated one-pot assay termed the One-pot TLAMP-PfAgo Assay (OTPA). This approach synergistically combines the rapid amplification capability of TLAMP with the sequence-specific detection precision of PfAgo, allowing for fast, sensitive, and specific identification of animal-derived DNA. Furthermore, OTPA eliminates intermediate handling steps, streamlining the workflow and minimizing the risk of amplicon contamination. By spatially isolating the reaction components with microcrystalline wax, we successfully integrated the two-step reaction into a one-pot closed tube. Crucially, while the specific assembly of this multi-layer structure involves precise pipetting steps during the development phase, the design is inherently engineered for industrial pre-fabrication. This allows the complex setup to be completed entirely during manufacturing, leaving the end-user with a simplified, ready-to-use detection tool.

2 Results and Discussion

2.1 Principle of one-pot TLAMP-PfAgo assay (OTPA)

To establish a rapid detection method, we chose TLAMP to amplify the target. TLAMP is a new variant of the LAMP reaction developed by Tang's group (Shi et al., 2024). The only difference between the TLAMP and

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traditional LAMP is the use of a turn-back loop primer (TLF/TLB) in the TLAMP. While such a modification is subtle, it gives TLAMP a higher amplification efficiency as more stem-loop amplicons can be generated in the TLAMP reaction. To avoid the unspecific amplification of TLAMP, we introduced the PfAgo-based specific signal reporting system to TLAMP. In this system, PfAgo can recognize and cleave the TLAMP amplicon using the artificially designed guide DNA. The cleaved amplicon then releases a new guide DNA that can couple with PfAgo to cleave the signal probe (labeled with fluorophore and quencher), producing a fluorescence signal. The workflow of the detection system is shown in **Fig. 1a**. The whole reaction is divided into three layers: the top layer is the TLAMP reaction for target amplification, the middle layer is the solid microcrystalline wax that can be melted when the temperature is raised above 80 °C, and the bottom layer is the PfAgo reaction. The TLAMP reaction and the PfAgo reaction are spatially isolated by the microcrystalline wax. Because the reaction temperature of TLAMP is 60 °C, the microcrystalline wax remains solid. After TLAMP, the temperature is raised to 95 °C to activate the PfAgo-based cleavage reaction. At such a high temperature, the microcrystalline wax melts into liquid form, resulting in the mixing of the TLAMP product and the PfAgo reaction. We named this detection system the One-pot TLAMP-PfAgo Assay (OTPA). With this design, the incompatibility between the TLAMP and PfAgo reaction could be bypassed, making the method simpler and reducing the risk of amplicon contamination from a lid-opening operation.

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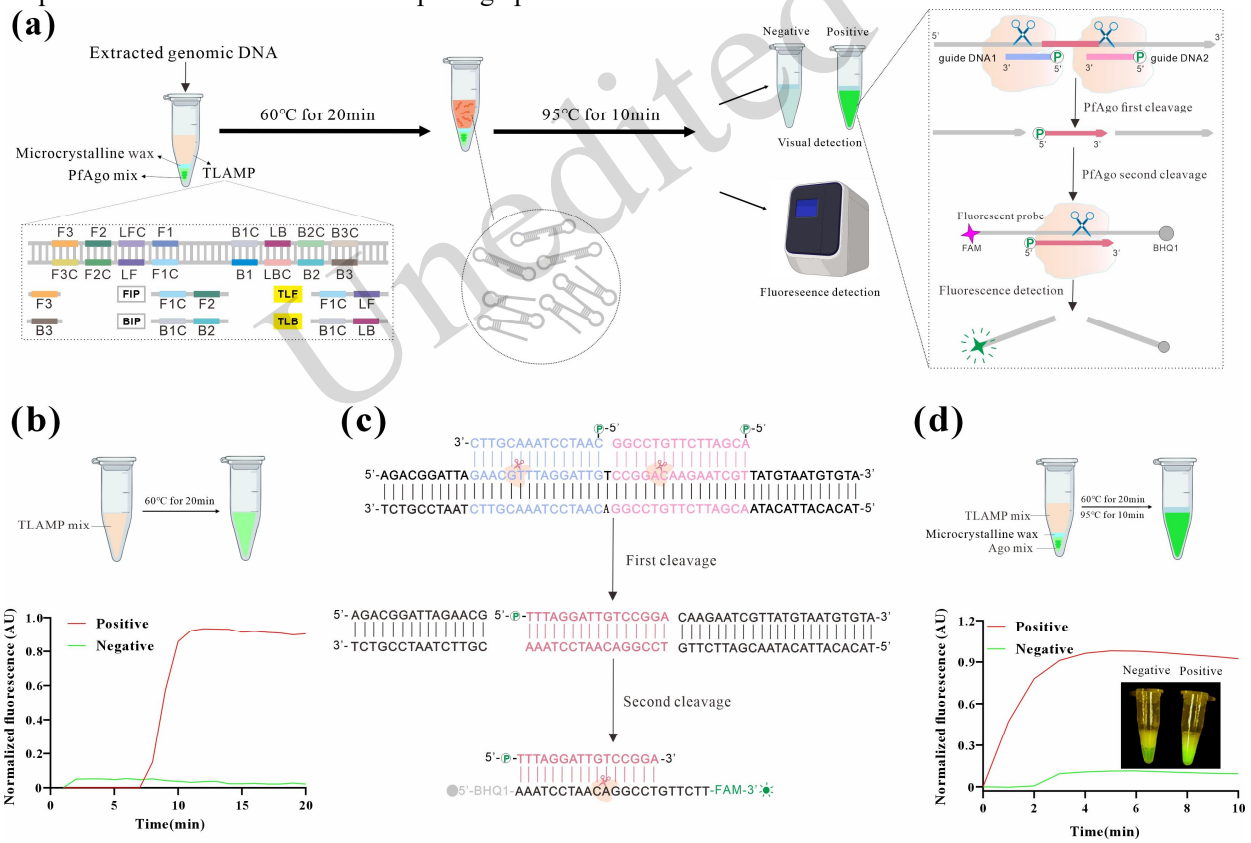

Fig. 1 The general principle and feasibility assay of One-pot TLAMP-PfAgo Assay (OTPA). (a) The workflow of OTPA. (b) The feasibility of TLAMP amplification for pork DNA. (c) The guide DNA design and sequence information of PfAgo-based sequence-specific detection. (d) The feasibility of the OTPA detection system.

2.2 Feasibility of the OTPA detection system

To evaluate the feasibility of the OTPA detection system, we selected pork as the model target, which is one of the most common substitutes used for meat adulteration. We designed a primer set based on the pig mitochondrial gene sequence. Then, we conducted a TLAMP experiment using SYBR Green I, a nonspecific

double-stranded DNA dye, to examine the primer and TLAMP. The positive reaction containing pork genomic DNA showed an enhanced fluorescence curve, indicating successful amplification of the target **(Fig. 1b)**. The negative control with no added sample DNA showed a flat fluorescence curve. This result confirmed the successful design of the primer and the establishment of the TLAMP assay for pork DNA detection. After that, we designed two guide DNAs according to the target sequence of amplification products to establish the PfAgo-based sequence-specific reporting. Two 15-nt guide DNAs with 5' end phosphorylation modification assembled with PfAgo exerted a first round of cleavage on the TLAMP amplicons, releasing a new guide DNA (**Fig. 1c)**. The new guide DNA induced a second round of cleavage by PfAgo, which cut the reporting probe and produced a fluorescence signal. To examine the guide design and PfAgo-based signal reporting, we initially conducted the classic two-step detection, in which the PfAgo solution was added to the inner side of the tube lid (Fig. S1a). The results in Fig. S1b verified the feasibility of the two-step reaction, demonstrating the design of guide DNA and PfAgo-based cleavage. To overcome the incompatibility between the TLAMP and PfAgo-based cleavage and simplify the operation, we carried out the OTPA reaction by using microcrystalline wax to physically isolate the TLAMP reaction and PfAgo reaction. Following a 20-min TLAMP reaction, the reaction temperature was raised to 95 °C to realize heat activation of the PfAgo reaction. The fluorescence intensity of the positive reaction showed a significant increase once the incubation temperature reached 95 °C, indicating the high cleavage efficiency of PfAgo (**Fig. 1d**). In contrast, the fluorescence intensity of the negative reaction showed negligible change. Denatured PAGE electrophoresis confirmed the cleavage of signal probe (Fig. S2). These results suggested the successful establishment of OTPA. Additionally, the endpoint fluorescence intensity under blue light (λ = 475 nm) also showed an obvious difference between the positive reaction and the negative reaction, implying the potential for visualized detection of the OTPA.

2.3 Optimization of the OTPA detection system

Having established the OTPA method, we sought to optimize the reaction by investigating several parameters affecting the OTPA analytical performance. Because the preamplification of target sequence was the rate-limiting step in the assay, we first optimized the TLAMP conditions to optimize amplification efficiency. In this experiment, the threshold time (Tt) was used as the indicator to evaluate TLAMP amplification efficiency. The fluorescence threshold was manually set at a fixed intensity value, which was determined to be significantly above the background baseline of negative controls to strictly exclude false signals. Consequently, the Tt value was defined as the precise reaction time point when the fluorescence intensity of the amplification curve crossed this pre-set threshold line. Changing the concentrations of outer primer (F3/B3) from 0.2 to 0.8 μmol/L did not induce a significant change in the Tt value ($P > 0.05$, **Figs. 2a** and S3a). Similarly, varying the concentration of the inner primer (FIP/BIP) (0.8 to 3.2 μmol/L) had little influence on TLAMP efficiency (**Figs. 2b** and S3b). These results indicate that the TLAMP reaction maintains high robustness across a relatively broad range of primer concentrations. In contrast, the concentration of turn-back loop primers (TLF/TLB) significantly affected reaction speed; increasing the concentration from 0.4 to 0.8 μmol/L significantly reduced the Tt value ($P < 0.05$, **Figs. 2c** and S3c), while increasing it further to 1.6 μmol/L yielded no significant improvement ($P > 0.05$). Mechanistically, these primers bind to the stem-loop region of the intermediate structures, providing additional starting points for DNA synthesis and strand displacement, thereby accelerating the exponential amplification phase significantly more than increasing the inner/outer primers alone. Consequently, 0.4 μmol/L (F3/B3), 1.6 μmol/L (FIP/BIP), and 0.8 μmol/L (TLF/TLB) was selected as the optimal primer set. Next, we optimized the enzyme and ion concentrations. The amplification speed increased significantly with Bst DNA polymerase dosage ($P < 0.05$, Fig. 2d). Since no statistically significant difference was observed between 0.4 U/μL and 0.5 U/μL ($P > 0.05$), 0.4 U/μL was chosen as the most cost-effective concentration. Regarding ionic conditions, the reaction achieved the lowest Tt values at 30-60 mmol/L for K^+ and 5 mmol/L for Mg^{2+} ions (**Figs. 2e**, **2f,** S3e and S3f). Notably, deviations from 5 mmol/L Mg^{2+} resulted in significantly delayed amplification ($P < 0.05$), highlighting the critical role of magnesium ions in enzymatic activity. Low concentrations are insufficient to catalyze the phosphodiester bond

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formation effectively, while excessive Mg^{2+} can stabilize non-specific primer annealing, potentially leading to background noise. Based on these data, the optimized conditions (0.4 U/μL Bst polymerase, 60 mmol/L K^+, and 5 mmol/L Mg^{2+}) were used for all subsequent OTPA assays.

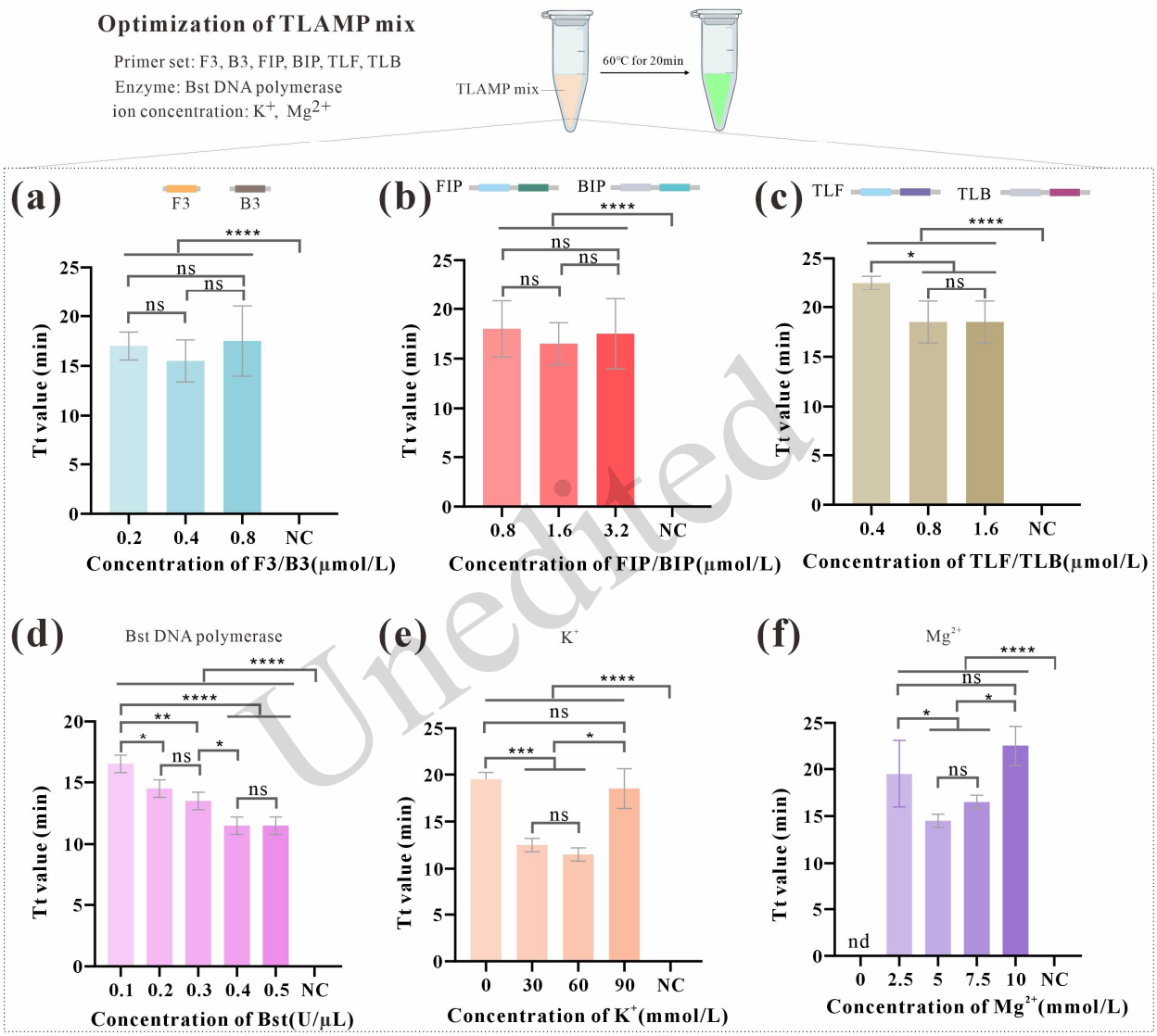

Fig. 2 Optimization of turn-back loop primer-accelerated LAMP (TLAMP). (a) Optimization of the concentration of the outer primer. (b) Optimization of the concentration of the inner primer. (c) Optimization of the concentration of the turn-back loop primer. (d) Optimization of the concentration of DNA polymerase. (e) Optimization of the concentration of K^+. (f) Optimization of the concentration of Mg^{2+}. Data are presented as mean ± SD from three independent replicates (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. Asterisks indicate significant differences between groups (* denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$, **** denotes $P < 0.0001$, ns denotes not significant, $P > 0.05$).

After optimizing the TLAMP reaction, we refined the conditions of the PfAgo-based cleavage reaction (Fig. S4). The number of guide DNA sequences was first examined. Only in the presence of two guide DNAs could the reaction yield a significantly increased fluorescence intensity (**Fig. 3a**), suggesting that two guide DNAs were essential for our method. We then optimized the concentration of guide DNA and found that 400 nmol/L of guide DNA was optimal to induce the strongest fluorescence signal (**Fig. 3b**). We inferred that a lower concentration of guide DNA may be insufficient to activate PfAgo, while a higher concentration may interfere with the assembly of PfAgo and the second-round guide DNA. Subsequently, we investigated the

amount of PfAgo. When the concentration of PfAgo was 0.3 μmol/L, the reaction produced the highest fluorescence intensity compared to the controls **(Fig. 3c)**. Unlike some pAgos that use Mg^{2+}, PfAgo is a thermophilic Argonaute that strictly depends on Mn^{2+} or Co^{2+} for its slicer activity. Mn^{2+} ions are required to coordinate within the catalytic tetrad (DEDD) of the PIWI domain to facilitate the hydrolysis of the phosphodiester backbone of the target DNA at high temperatures. As depicted in **Fig. 3d**, 1.4 mmol/L of Mn^{2+} was selected for the following experiments. Based on all these findings, we obtained the optimized conditions for OTPA (Table S2).

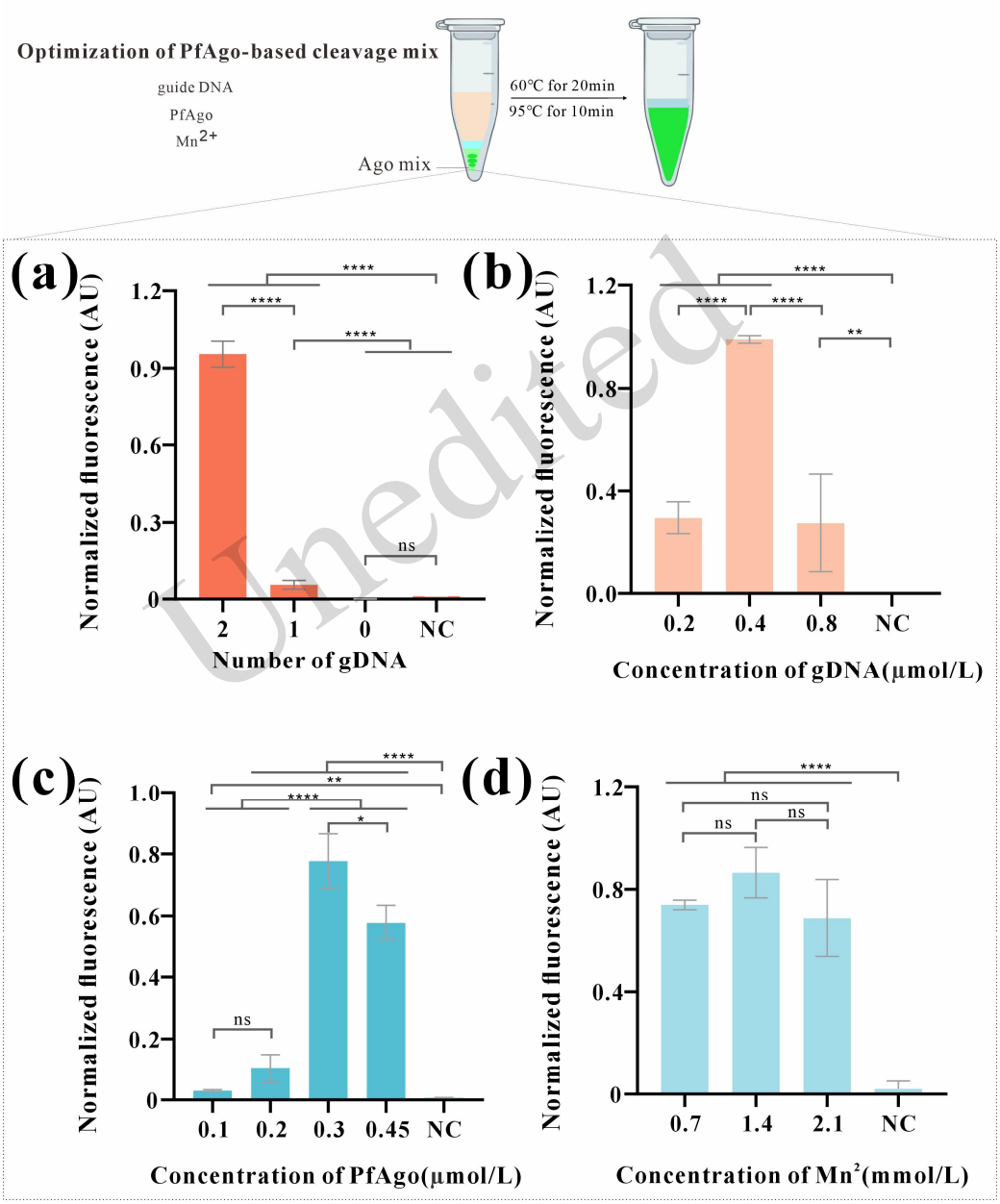

Fig. 3 Optimization of PfAgo-based cleavage in One-pot TLAMP-PfAgo Assay (OTPA). (a) Optimization of the number of guide DNA sequences. (b) Optimization of the concentration of guide DNA. (c) Optimization of the concentration of PfAgo. (d) Optimization of the concentration of Mn^{2+}. Data are presented as mean ± SD from three independent replicates (n=3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. Asterisks indicate significant differences between groups (* denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$, **** denotes $P < 0.0001$, ns denotes not significant, $P > 0.05$).

2.4 Analytical performance of OTPA

To assess the analytical performance of OTPA, we used a series of 10-fold diluted pork genomic DNA templates ranging from 3×10^{-1} to 3×10^{-6} ng/μL as the input. The OTPA assay could sensitively detect as low as 3×10^{-4} ng/μL of pork DNA **(Fig. 4a)**. We also conducted the visualized OTPA assay under blue light to assess its potential for on-site detection with a handheld blue light illuminator. Notably, sensitivity was comparable to or even better than that of many reported methods (Perestam et al., 2017; Hardianto et al., 2023; Fang et al., 2025). This could be ascribed to the efficient amplification of TLAMP and the specific signal expansion of PfAgo. Besides, the detection time of the assay was 30 min, which represented a significant advantage over current methods. To evaluate the specificity of the OTPA assay, we tested genomic DNA from a comprehensive panel of meat species, including the target (pork) and non-targets (beef, chicken, duck, rabbit, sheep, and horse). Only the reaction containing pork DNA triggered a significant increase in fluorescence intensity ($P < 0.0001$, **Figs. 4b** and S5). Conversely, all non-target species exhibited fluorescence levels comparable to the negative control (NC), with no significant differences ($P > 0.05$). To further investigate the specificity mechanism and the tolerance of PfAgo to incorrect sequences, we performed a challenge test using mismatched guide DNAs. As shown in Fig. S6, only the system with perfectly matched guide DNAs generated a significant fluorescence signal. In contrast, the group with mismatched guide DNAs showed a low background signal comparable to the negative control (NC). This result provides strong evidence that the PfAgo cleavage is strictly sequence-dependent, acting as a critical gatekeeper to prevent false-positive signals from non-specific amplification products. This confirms that the OTPA has high specificity and can distinguish pork from other common mammalian and avian meats. We further examined the feasibility of our method for detecting pork meat within a beef mixture at various concentrations. A positive signal was observed even when the pork meat constituted only 0.1% of the mixture, validating the high selectivity of our method **(Fig. 4c)**. To further challenge the robustness of the OTPA against interference from non-target genomic DNA, we simulated adulteration scenarios using chicken and mixed poultry (chicken and duck) as background matrices. The assay showed high specificity and sensitivity in these complex matrices (Fig. S7). Significant fluorescence signals were consistently detected in samples containing 1% or 0.1% pork ($P < 0.0001$), demonstrating that the abundant background DNA from chicken and duck did not inhibit the target amplification. The fluorescence signals for the 0.01% spiked samples were indistinguishable from the negative control ($P > 0.05$), determining the practical limit of detection (LOD) for pork adulteration in mixed meat products to be about 0.1%. Note that adulteration levels below 0.1% in meat products are typically regarded as accidental contamination (Yang et al., 2014), suggesting that the sensitivity of the OTPA is adequate for identifying pork adulteration. To examine the stability of the microcrystalline wax, prefabricated reaction tubes containing microcrystalline wax were stored at 4 °C (2 months), room temperature (25 °C, 1 week), and 37 °C (3 days). Freshly prepared reaction tubes served as controls to detect pork DNA (3×10^{-4} ng/μL). The results shown in Fig. S8 indicated that the intensity of the fluorescence signals in each storage group and the control group were similar. This consistency not only confirms the long-term stability of the reagents but also demonstrates the robustness of the wax-based separation system. Specifically, the successful signal generation across all conditions verifies that the wax layer melts completely and reagents mix efficiently at 95 °C, unaffected by batch preparation or storage history. We evaluated the performance of the OTPA in comparison with previously reported PfAgo-based methods. Our method exhibited comparable sensitivity and high specificity, attributed to the robust signal amplification facilitated by PfAgo-based cleavage (Table S4). Furthermore, the entire detection process could be completed within 30 min in a single reaction tube, rendering this method an attractive option for practical applications. Additionally, the simplicity of the method allows for its adaptation to high-throughput food screening, which is advantageous for food composition authentication. In summary, the OTPA has high sensitivity and specificity, a short detection duration, ease of operation, and visual readout, demonstrating great potential for future POCT applications, particularly in decentralized settings.

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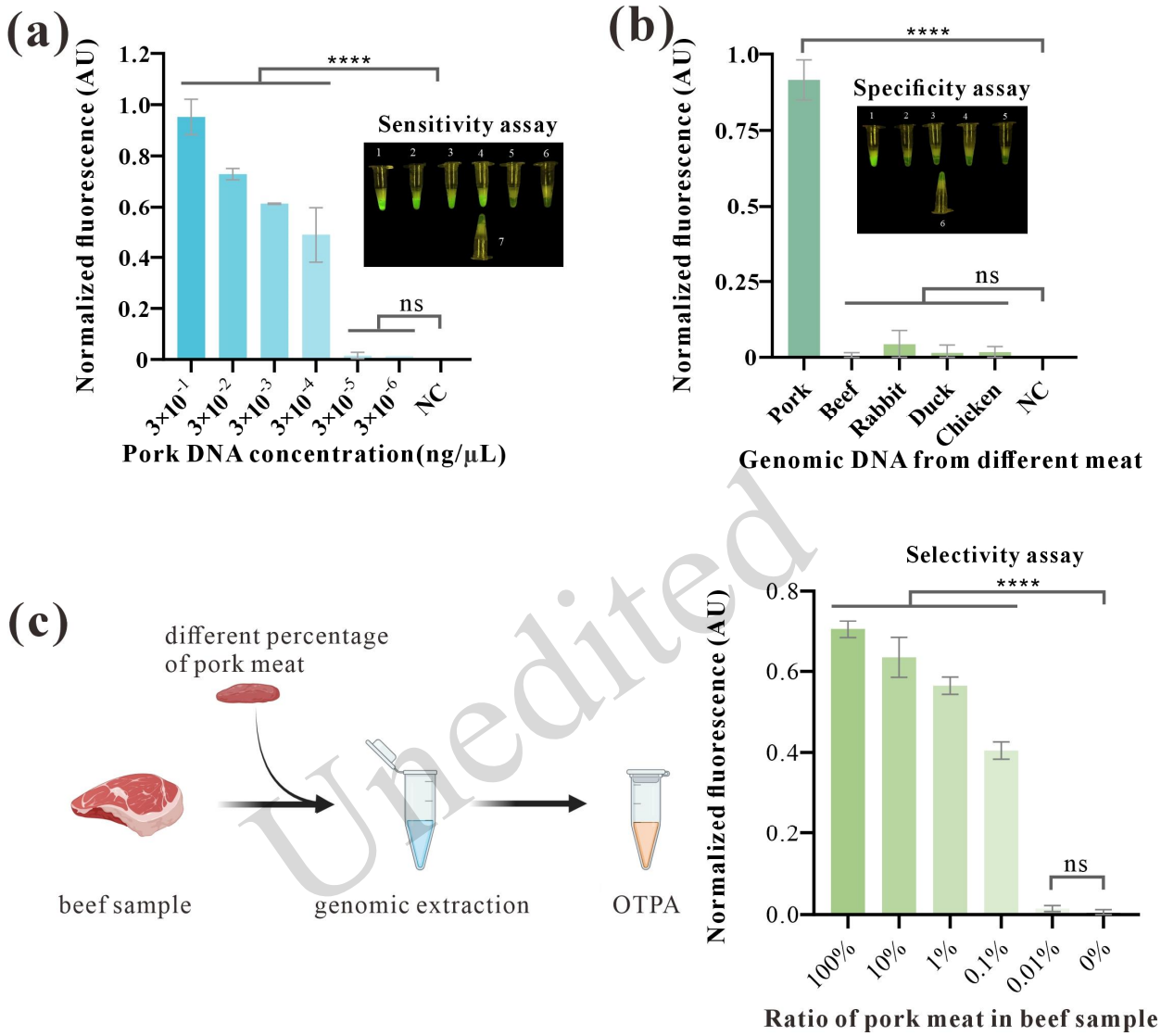

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Fig. 4 Evaluation of the sensitivity and specificity of One-pot TLAMP-PfAgo Assay (OTPA). (a) End-point fluorescence values of the OTPA when detecting serially diluted pork genomic DNA. The visualized OTPA assay under blue light. The reaction tube 1-6 represents the sample DNA concentration of 3×10^{-1} ng/μL - 3×10^{-6} ng/μL, and tube 7 was the negative control with only water added. (b) Specificity assessment of the OTPA when detecting pork DNA. The genomic DNA concentration of pork, beef, rabbit, duck, and chicken was 0.3 ng/μL, 0.2 ng/μL, 0.5 ng/μL, 3 ng/μL, 1.1 ng/μL, respectively. The visualized OTPA under blue light. Reaction tube 1-5 represents the sample DNA of pork, beef, rabbit, duck, and chicken; tube 6 was the negative control with only water. added (c) Evaluation of the selectivity of the OTPA by detecting a beef sample mixed with different ratios of pork meat (100%, 10%, 1%, 0.1%, 0.01%). Endpoint fluorescence data are expressed as mean ± standard deviation (SD, n=3). NC: Negative Control (ddH_2O). Statistical significance was determined by Student's t-test (**** indicates $P < 0.0001$ vs. NC, ns denotes no significance, with $P > 0.05$).

2.5 Establishment of duplex OTPA

Unlike the non-specific trans-cleavage activity of the most-used CRISPR/Cas systems, PfAgo-based cleavage was sequence-specific, ideal for developing a one-pot multiplex detection method. PfAgo exerts cleavage activity only when it forms a complex with a 5'-phosphorylated guide DNA and specifically binds to a target sequence complementary to the guide DNA. Unlike CRISPR/Cas systems (which have non-specific trans-cleavage), PfAgo relies strictly on guide DNA-target base pairing. This intrinsic property eliminates

non-specific cleavage and is the core of duplex compatibility. To prove the concept, we attempted to develop a duplex OTPA assay by detecting pork and beef simultaneously. We rationally designed the TLAMP primer sets, specific probe, and guide DNAs based on the beef genomic DNA sequence. In our study, the duplex systems used species-specific primers (T-P- vs. T-B- series, Table S1) and guide DNAs (g-P- vs. g-B- series, Table S1), paired with differential fluorophores (FQ-P-Fam for pork, FQ-B-Rox for beef; Table S1). After that, we successfully established the TLAMP reaction for beef DNA detection (Fig. S9a). Then, a singleplex OTPA for beef DNA was also developed (Fig. S9b). The OTPA could detect down to 2×10^{-4} ng/μL of beef genomic DNA with good specificity (Fig. S10). Next, we combined the TLAMP primer sets of pig and cattle and four guide DNAs in one OTPA reaction and input different templates. Note that the FAM-labeled probe was designed for pork DNA and ROX-labeled probe was designed for beef DNA. After 20 mins of TLAMP reaction, we heated the reaction to 95 °C. When adding the mixed templates (pork + beef), both the fluorescence channels showed an enhanced fluorescence intensity **(Fig. 5)**. Besides, adding pork or beef alone could induce the positive signal only in the FAM channel or ROX channel, and no interference between the templates was observed. In contrast, the negative control showed unchanged fluorescence signal in both channels. The visualized OTPA also confirmed the result (Fig. S11). These results indicated the successful development of the duplex-OTPA, demonstrating the feasibility of one-pot multiplex detection of our method. Compared with the singleplex assay, the duplex OTPA showed significant advantages, such as high efficiency, cost-saving, and practicality (Table S3). Crucially, the reliability of negative results is a major concern in food safety testing, as enzyme inactivation or inhibitor presence could lead to false negatives. The duplex OTPA system inherently addresses this issue by serving as a self-validating platform. In a typical adulteration scenario of pork in beef meat, the amplification signal of the dominant meat matrix (beef, ROX channel) acts as an internal amplification control (IAC). A positive signal in the IAC channel confirms that the reaction conditions are optimal. Therefore, if a sample presents a positive background signal (beef) but a negative target signal (pork), the negative result can be confidently interpreted as the absence of adulteration rather than a system failure. Note that the duplex OTPA has the potential to expand multiplexity once the primer sets and guide DNAs are rationally designed. However, performance is constrained by the TLAMP step, where each target requires six primers. Adding more than three targets increases primer-dimer formation and competition for reagents. Future improvements, such as using microfluidic chips to separate amplification reactions while combining PfAgo signal detection, could overcome this limitation and enable higher-throughput analysis.

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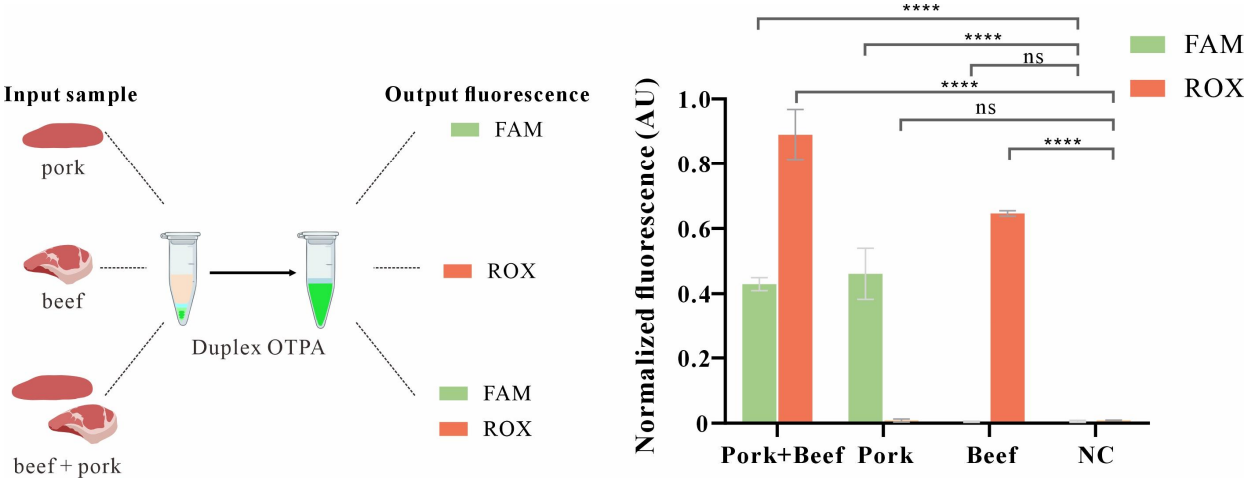

Fig. 5 Establishment of the duplex One-pot TLAMP-PfAgo Assay (OTPA) for pork and beef detection. The amounts of pork DNA and beef DNA were 3×10^{-2} ng/μL and 2×10^{-2} ng/μL, respectively. Endpoint fluorescence data are expressed as mean ± standard deviation (SD, n=3). NC: Negative Control (ddH_2O). Statistical significance was determined by Student's t-test (**** indicates $P < 0.0001$ vs. NC, ns denotes no significance, with $P > 0.05$).

2.6 Detection of commercial samples using duplex OTPA

Accurate and rapid authenticity detection of meat plays a vital role in ensuring food safety. To evaluate our method in practical use, we collected and tested 15 commercial meat products, including mixed meat products and deep-processed products, from a local market. Then we proposed a workflow (**Fig. 6a**) to test these samples with our method. The positive control group (adding pork and beef DNA) triggered the fluorescence signal in both channels **(Fig. 6b)**. The DNA samples extracted from pork slices, pork sausage, pork-chicken mixed meatballs, and canned meats induced increased fluorescence signal only in the FAM channel, confirming that these commercial products comprise pork. In contrast, the DNA extracted from beef sausage, spiced beef, beef slice, and beef paste exclusively triggered fluorescence signals in the ROX channel, indicating the presence of beef DNA in these products. These results indicated that there was no adulteration in the ingredients of these tested meat products, which was consistent with the standard real-time fluorescence PCR method (**Fig. 6c**). Given that deep-processed products typically undergo harsh thermal treatments, such as high-pressure sterilization that inevitably lead to genomic DNA fragmentation, valid concerns may arise regarding the impact of DNA integrity on detection performance. To rigorously address this and validate the robustness of our assay against DNA degradation, we simulated these conditions by subjecting raw meat to prolonged boiling (100 °C for 60 min) and autoclaving (121 °C for 20 min). Despite the severe processing conditions known to fragment DNA, the OTPA platform successfully generated distinct positive fluorescence signals in all simulated groups (Fig. S12). Note that the fluorescence intensity of the autoclaved group was slightly lower than that of raw meat, which is consistent with the reduction in intact templates, but it remained sufficient for clear identification. These results mechanistically support our successful detection of commercial canned samples (Fig. 6b), confirming that the short-amplicon design of OTPA ensures reliable performance even with low-integrity DNA. To further substantiate the applicability of our method, we used the duplex OTPA to analyze 20 commercial beef samples of which 7 samples (samples 1, 5, 6, 9, 11, 14, 18) were artificially contaminated with pork. As shown in **Figs. 6d** and **6e**, all samples exhibited increased fluorescence signals in the ROX channel, confirming the authenticity of the beef samples. Additionally, the seven spiked samples showed a significant fluorescence signal in the FAM channel, demonstrating the presence of pork adulteration. This result was verified by a real-time PCR assay using the corresponding authentication primer sets (Fig. S13). Taken together, these results suggest that our method holds potential as a dual-purpose tool for detecting pork adulteration and verifying beef authenticity, which is promising for food safety testing. Overall, the data underscore the feasibility of the duplex OTPA for pork and beef authentication, highlighting its potential for practical application.

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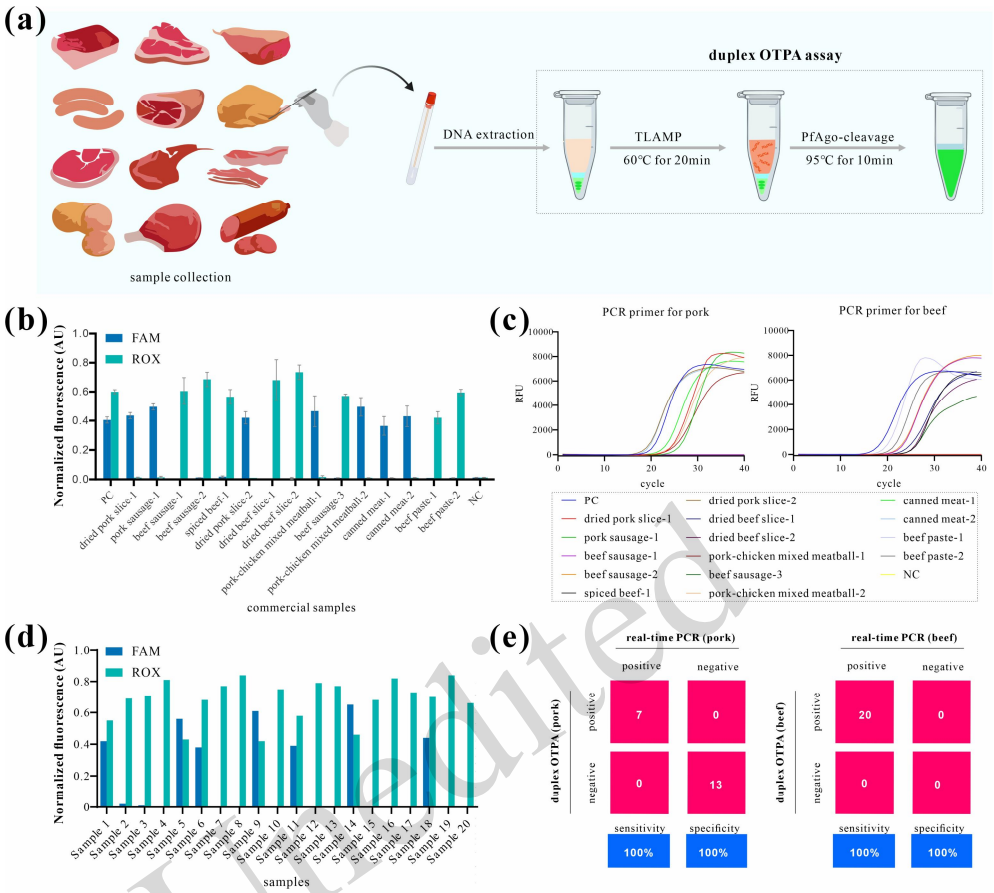

Fig. 6 Validation of the duplex One-pot TLAMP-PfAgo Assay (OTPA) method using commercial meat products. (a) a schematic illustration of the workflow using duplex OTPA to detect commercial products. (b) Endpoint fluorescence detection using duplex OTPA for different meat products. The values are expressed as mean±standar deviation (SD), n=3. (c) Real-time fluorescence PCR-based validation of different meat products. (d) Application of the duplex OTPA for double-blind screening of 20 commercial beef samples. Samples 1, 5, 6, 9, 11, 14, and 18 were artificially spiked with pork. The detection results for all 20 samples showed 100% concordance with the parallel validation performed using real-time PCR (Fig. S10). (e) Evaluation of the sensitivity and specificity of the duplex OTPA for pork and beef was done by comparing to real-time PCR results using a confusion matrix.

3 Conclusions

In this study, we successfully developed and validated a novel, integrated biosensing platform, OTPA, for rapid, sensitive, and specific authentication of meat species. The core innovation of this system lies in its intelligent design, which synergistically combines the rapid amplification capability of TLAMP with the precise sequence-specific recognition of PfAgo. A key feature is the incorporation of a heat-activatable microcrystalline wax barrier that spatially and temporally compartmentalizes the amplification and detection reactions within a single closed tube. This elegantly simple yet highly effective strategy overcomes the longstanding challenge of enzymatic incompatibility, thereby eliminating the need for post-amplification handling and significantly reducing the risk of aerosol contamination. The OTPA platform shows exceptional analytical performance, achieving detection limits as low as 3×10^{-4} ng/μL for pork DNA and 2×10^{-4} ng/μL for beef DNA within 30 min. The assay exhibits high specificity, enables duplex target detection, and supports direct visual interpretation of results under blue light illumination. These attributes collectively underscore its strong potential for POCT applications. The practical utility and reliability of the OTPA method were rigorously confirmed through the analysis of commercial meat products, which showed perfect concordance with a standard PCR qualitative method. Compared to CRISPR/Cas-based diagnostics, the OTPA system offers

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distinct trade-offs and advantages. First, in the cleavage mechanism, CRISPR/Cas enzymes like Cas12a rely on promiscuous trans-cleavage for signal amplification, which limits single-tube multiplexing by causing indiscriminate reporter probe degradation. In contrast, PfAgo enables precise sequence-specific cleavage, preventing crosstalk and allowing robust one-pot multiplex detection, despite lacking collateral amplification. Second, PfAgo operates at 95 °C, requiring thermal separation methods to protect DNA polymerase. However, this high temperature enhances hybridization stringency, greatly reducing non-specific signals common in lower-temperature CRISPR assays. Third, OTPA uses short DNA guides, which are more stable, cheaper, and easier to produce than the long, fragile RNA guides needed for CRISPR, making OTPA an appealing choice for POCT use.

Regarding temperature control, unlike PCR which requires precise rapid cycling, the OTPA workflow demands only two static temperature blocks. This can be easily achieved using battery-powered portable dry baths or handheld isothermal heaters widely found in current field laboratories. The robustness of PfAgo means that the high-temperature step serves mainly to melt the wax and activate the enzyme, a process that tolerates minor temperature fluctuations inherent in portable devices. Furthermore, for quantitative analysis, smartphone-based portable fluorometers or miniature fluorescence detectors are fully compatible with our reaction tubes. Therefore, the OTPA system requires no custom hardware development and can be directly integrated with existing off-the-shelf portable instrumentation for immediate field deployment. Another practical concern regarding the OTPA is the apparent complexity of preparing the three-layer reaction system, which seems time-consuming compared to simple reagent mixing. However, the OTPA system is designed for mass-scale pre-fabrication. As validated by our stability tests, the reaction tubes containing the PfAgo layer and wax barrier can be pre-assembled and stored for extended periods without loss of activity. For the end-user, the workflow is therefore streamlined to a simple procedure, eliminating the burden of a complex setup stage. From an economic perspective, despite the manufacturing steps, the overall cost remains competitive (Table S5). The assay uses short, unmodified DNA guides and standard primers, which are significantly less expensive than the components of many commercial isothermal detection methods. Furthermore, the rapid turnover time (30 min total detection) significantly reduces labor costs in high-throughput screening scenarios, balancing the initial manufacturing effort.

In summary, the OTPA system represents a significant advancement in the field of intelligent biosensing for food authentication. It provides a robust, user-friendly, and cost-effective tool that is ideally suited for on-site food safety surveillance, effectively addressing the critical need for rapid and reliable detection of meat adulteration. Beyond its immediate application, the modular design principle of the OTPA platform, where target specificity is determined by the choice of primers and guide DNAs, holds considerable promise for broad adaptability. This platform can be readily reused for the detection of a wide array of biological targets, including foodborne pathogens, veterinary drug residues, and genetically modified organisms, thereby substantially expanding its impact in the realms of food safety, biomedical diagnostics, and environmental monitoring. The work presented herein not only offers a practical solution for combating global food fraud but also provides a versatile and powerful technological framework for the future development of next-generation, field-deployable biosensors.

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Materials and methods

The detailed materials and methods used in the study were listed in the Supporting Information.

Data availability statement

The data used to support the findings of this study are included within the article and its supplementary materials. No additional datasets were generated or analysed during the current study.

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Author contributions

Sheng DING performed the experimental research and data analysis, wrote and edited the manuscript. Jing LI performed the experimental research and data analysis, wrote and edited the manuscript. Minglong Yang performed the experimental research and data analysis, wrote and edited the manuscript. Yujia Song performed the experimental research and data analysis. Xiangke NIU performed the experimental research and data analysis. Mei LI contributed to the study design, data analysis, writing and editing of the manuscript. Yao LUO contributed to the study design, data analysis, writing and editing of the manuscript. All authors read and approved the final manuscript and, therefore, had full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

The authors declare that they have no conflict of interest.

Declaration on the use of generative AI tools

The authors declare that no generative AI tools were used in the creation of this manuscript. All content, analysis, and writing were performed solely by the authors.

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Supplementary information:
Materials and methods; Table S1-S5; Figs. S1-S13

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